**Supplemental Material**

# Renal Denervation Prevents Immune Cell Activation and Renal Inflammation in Angiotensin II–Induced Hypertension

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**Detailed Materials and Methods**

**Animals:** All mice were obtained from Jackson Laboratories on a C57Bl/6 background. In the case of the β2AR-/- mice, these were outbred from β1/β2AR$^{-/-}$ mice. At 3 months of age, mice were randomly selected for renal denervation or sham surgery For renal denervation, mice were anesthetized by intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg) and the renal arteries were visualized via flank incisions. The renal nerves were ablated by encircling the renal artery with a 5-0 suture soaked with 10% phenol in ethanol. In sham-operated mice, normal saline was applied rather than phenol. In some experiments, unilateral denervation was performed by applying phenol to only the left renal artery. To selectively disrupt renal afferent nerves, a similar surgical approach was used except capsaicin (33 mmol/L) dissolved in 5% Tween 80, 5% ethanol and 90% normal saline was applied to the renal artery for 15 minutes as described recently[1]. Blood pressure was measured either invasively using telemetry or non-invasively using an automated tail-cuff system as previously described.[2] Hypertension was induced by the infusion of angiotensin II (490 ng/kg/min) via osmotic minipumps for two weeks to four weeks. At study termination, mice were euthanized by exposure to $CO_2$. One week later, osmotic minipumps were implanted subcutaneously for infusion of angiotensin II (490 ng/kg/min) or vehicle for 2 weeks unless otherwise indicated. In subsets of mice, telemetry units were implanted for measurement of blood pressure. After at least one week recovery from telemetry implant, blood pressure was recorded for 10 minutes every hour for the duration of the experiments (i.e. three days prior to osmotic minipump implantation and until the end of angiotensin II infusion at Day 14). At the end of each experiment, mice were sacrificed with $CO_2$ inhalation and the chest was rapidly opened and the superior vena cava sectioned. A catheter was placed in the left ventricular apex and the animals were perfused at a physiological pressure with KrebsHepes buffer until the effluent from the vena cava was cleared of blood. The Institutional Animal Care and Use Committee approved all experimental protocols.

**Material** The antibodies and fluorophores were purchased from Biolegend (San Diego, CA) included: 7-AAD for live/dead staining; BV510-conjugated anti-CD45 (30-F11); APC-conjugated anti-CD4 (\GK1.5); APC/Cy7-conjugated anti-CD8 (53-6.7); PE/Cy7-conjugated anti-CD3 (145-2C11); FITC-conjugated CD44 (IM7); PE-conjugated anti-CD80 (16-10A1); BV421-conjugated anti-CD86 (GL-1); PE/Cy7-conjugated anti-I-A$^b$ (AF6-120.1);APC/Cy7-conjugated anti-CD11c (Bu15), APC-conjugated anti-CD11b (M1/70), FITC-conjugated anti-B220 (RA3-6B2). An Alexa 488 tagged single-chain antibody that has been previously described was used for detecting intracellular isoketal adducts D11.[3] Primary antibodies for Western blot included rabbit polyclonal antibodies anti-tyrosine hydroxylase (AB152 from Millipore), rabbit monoclonal anti-$\alpha_{2B}$ adrenergic receptor (ab151727), anti-$\beta_1$ adrenergic receptor (ab3442), anti-$\beta_2$ adrenergic receptor(ab182136, all from Abcam) and anti-GAPDH (sc-32233, from Santa Cruz Biotechnology). Enzyme immunoassay kits for urinary albumin and nephrin were purchased from Exocell (Philadelphia, PA). Enzyme immunoassay kit for calcitonin gene related peptide was fromCayman Chemical (Ann Arbor, MI). All primers and probes for real time PCR (NOS3: Mm00435217_m1, Klf4: Mm00516104_m1, MCP-1/Ccl2: Mm00441242_m1, VCAM-1: Mm01320970_m1, ICAM-1: Mm00516023_m1 gene expression assays as well as a GAPDH endogenous control) were from Applied Biosystems. Norepinephrine and neuropeptide Y were purchased from Sigma Aldrich (St Louis, MO).

**Measurements of Albuminuria/Nephrinuria:** Albumin and nephrin were measured by ELISA from 24 hour urine samples as described previously.[3] All concentrations were multiplied by total urine volume to obtain the daily excretion rate.

**Catecholamines:** After euthanasia, the kidneys and adrenal glands were freeze-clamped and pulverized by a mortar and pestle chilled in liquid nitrogen. Norepinephrine and epinephrine contents were measured by high-performance liquid chromatography via electrochemical detection as previously described.[4]

**Calcitonin Gene Related Peptide:** With kidneys harvested immediately after euthanasia, the renal pelvis using a dissecting microscope. Tissues were homogenized in 1M acetic acid and CGRP was measure according to the protocol provided in the ELISA kit.

**Western Blot Analysis and Real-time PCR:** Western blotting was performed using antibodies against tyrosine hydroxylase, $\alpha_{2B}$, $\beta_1$ and $\beta_2$ adrenergic receptors, and GAPDH. Goat anti-rabbit and goat anti-mouse secondary polyclonal antibodies were employed. Western blots were quantified by densitometry. Levels of endothelial nitric oxide synthase (eNOS), transcription factor Kruppel-like factor 4 (Klf4), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), regulated on activation normal T cell expressed and secreted (RANTES), and monocyte chemotactic protein 1 (MCP-1) mRNA were measured using TaqMan real-time PCR.

**Cytokine Detection:** DCs were positively selected from the spleen using an autoMACS separator and CD11c magnetic beads (Miltenyi Biotech). The purity of these was confirmed to be >95% by flow cytometry. Splenic $CD11c^+$ cells were placed in 96-well plates at a density of $5\times10^5$ per well and cultured in 200 µL RPMI1640 medium supplemented with 10% fetal bovine serum for 24 hours. Cytokines released into the medium were quantified via a Luminex assay. We have previously confirmed that this kit does not select mouse macrophages.[3]

**Confocal Microscopy:** For superoxide detection, kidneys were rapidly removed after euthanasia, immersed in optimal cutting temperature (OCT) media and frozen in dry ice. Thirty µm sections were obtained and used for detecting superoxide by DHE as described previously.[5] Sections were imaged using confocal microscopy with an excitation wavelength of 405 nm and an emission wavelength of 530-560 nm. For β3 tubulin staining, lymph nodes adjacent to the kidneys were carefully isolated, and 10-µm-thick frozen sections were prepared. Images were obtained using a primary anti-β3 tubulin antibody and the Alexa 488 goat anti-rabbit secondary antibody.

**Dendritic Cell Adoptive Transfer:** One million splenic DCs were obtained from mice that had undergone either sham surgery or bilateral renal denervation and subsequent angiotensin II or vehicle infusion for two weeks. These were suspended in 200 µL PBS, and adoptively transferred to naïve mice by tail vein injection. Telemetry transmitters were implanted in the recipient mice one week before adoptive transfer, and low dose (140 ng/kg/min) angiotensin II infusion were initiated 10 days later.

**Preparation of Bone Marrow Derived Dendritic Cells:** Bone marrow cells from C57BL/6 and $\beta2AR^{-/-}$ mice were cultured in 6-well plates at a concentration of $0.5\times10^6$ cells/ 2.5 ml in the presence of GMCSF and IL-4 as described previously.[6] Norepinephrine (1 – 3 µmol/L) was added to the medium at the beginning of culture, and supplemented on day 3 and day 6. In other experiments neuropeptide Y was added to achieve a final concentration of 100 nmol/L.

**Immunohistochemistry:** Five micron sections were obtained from formalin fixed, paraffin embedded kidneys. Collagen was visualized by Masson Trichrome blue staining, and immunohistochemistry was used to detect CD3+ and F4/80+ cells in the kidney as previously described.[3, 7]

**Magnetic Resonance Imaging:** Relative renal blood flow (rRBF) and relative renal blood volume (rRBV) were measured by dynamic susceptibility contrast magnetic resonance imaging (DSC-MRI) with the administration of iron oxide nanoparticles as described.[8, 9] Six mice with unilateral renal denervation and two-week angiotensin II infusion were used in this experiment. One or two days before of scanning, a catheter was placed in the jugular vein, and externalized from the interscapular region for administration of contrast agent. Mice were anesthetized (isoflurane 1.5-2%) and scanned on a Varian 7T MRI system using a Doty 38 transmit/receive coil. Body temperature was maintained at 37° during the scan. An oblique axial plan through both kidneys was chosen. A dynamic susceptibility-weighted gradient-echo sequence was applied during an iv injection of monocrystalline iron oxide nanoparticles (MION, 6mg/kg), which was administered rapidly in less than one second. A dynamic series of 600 images (~1s/volume) was acquired up to 10 minutes. $R_2$ imaging was performed using a multiple spin echo sequence with refocusing pulses of 180 (repetition time=2500ms, 16 echoes, 4 averages) before and after MION injection. The averaged $\Delta R_2$ across voxels of each kidney was used to represent rRBV, while peak amplitude/area from the relative DSC signal intensity time curve of each kidney was used to represent rRBF ($sec^{-1}$).

**Statistics:** Data in the manuscript are expressed as mean ± SEM. For telemetry and tail cuff blood pressure measurements over time, two-way ANOVA with repeated-measures was employed, followed with a Bonferroni post hoc test when significance was indicated. To compare the effect of renal denervation on renal inflammation, two-way ANOVA was used as indicated. Because of differences in variation between groups, we employed a non-parametric rank sum test with a Bonferroni correction for comparisons of cytokine release by DCs. P values are reported in the figures and a value less than 0.05 was considered significant.

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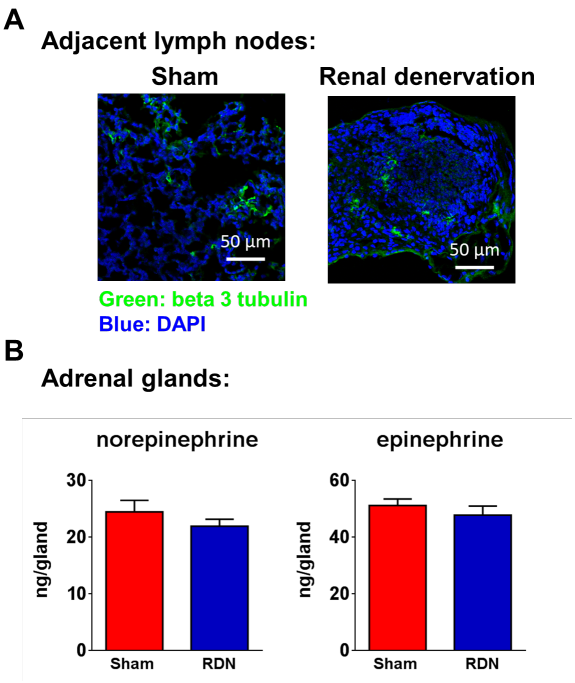
**Supplemental Table I: Effect of Renal Denervation on Cytokine Production in DCs**

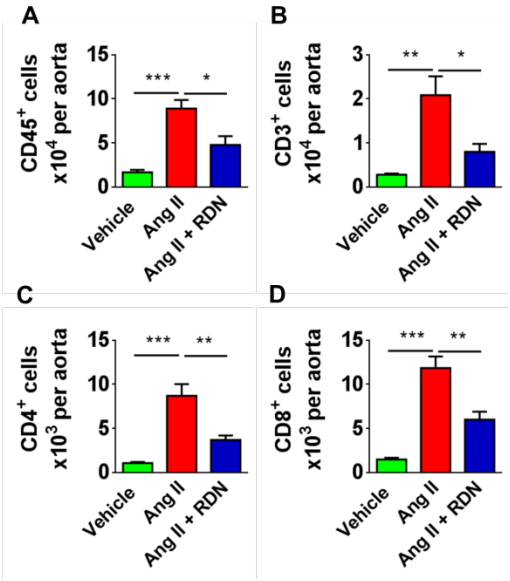
| Cytokine | Sham (n=5) | Ang II (n=8) | Ang II + RDN (n=8) |
|---|---|---|---|
| IL-2 | 0.65±0.06 | 1.87±0.42 * | 1.28±0.35 |
| IL-12 p40 | 0.00±0.00 | 0.22±0.18 | 0.00±0.00 |
| IL-12 p70 | 0.40±0.13 | 0.84±0.24 | 0.39±0.09 |
| IL-23 | 820.4±328.16 | 672.2±245.6 | 377.6±60.62 |
| GM-CSF | 13.9±3.46 | 76.04±35.65 | 13.65±3.60 |
| TGFβ1 | 1.80±0.16 | 1.87±0.22 | 2.27±0.29 |
| TGFβ2 | 1.75±0.14 | 1.70±0.05 | 1.88±0.10 |
| TGFβ3 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |

In pg per $10^6$ cells. *p<0.05 vs. Sham.

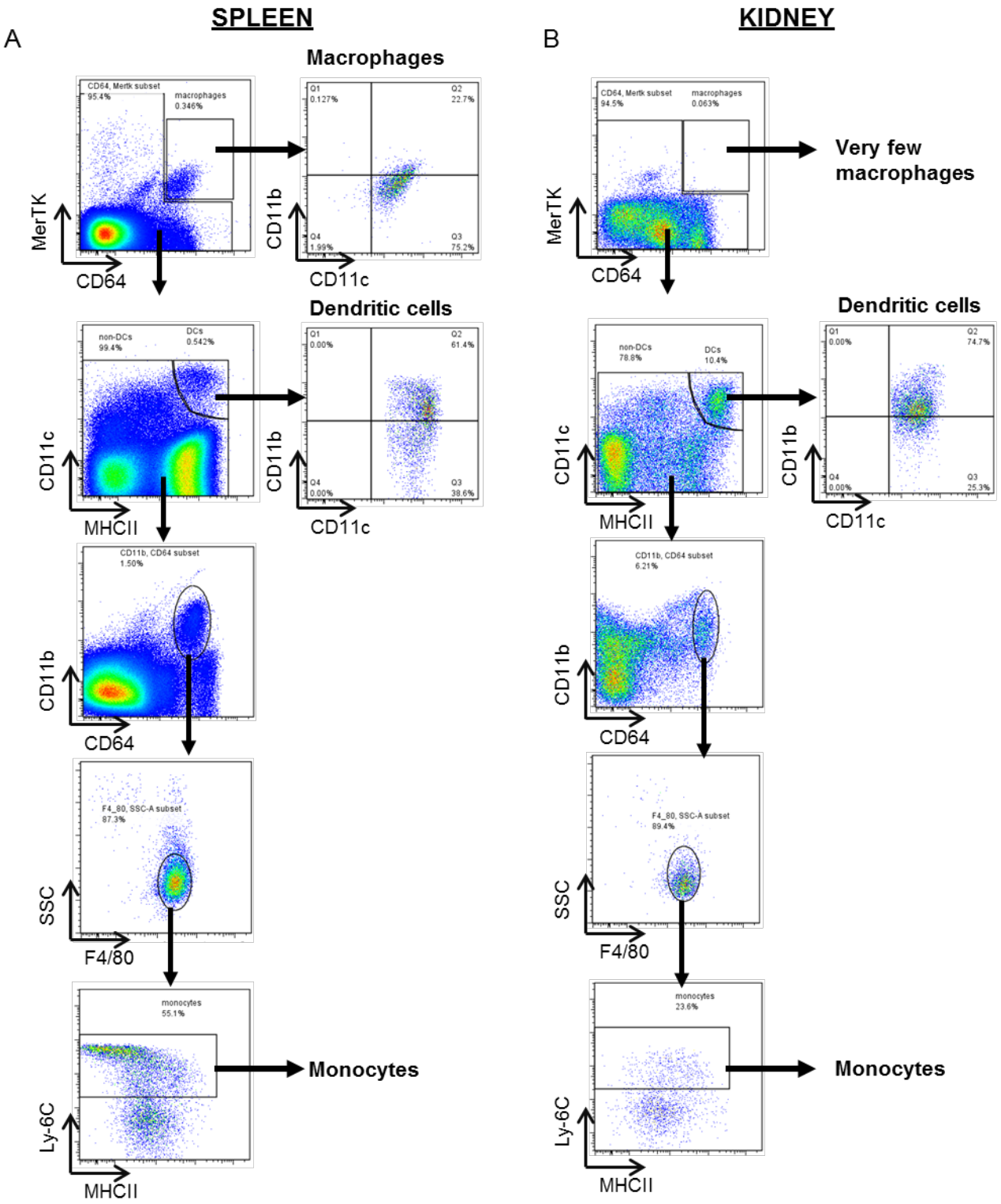
IL: interleukin
GM-CSF: granulocyte-macrophage colony-stimulating factor
TGF: transforming growth factor



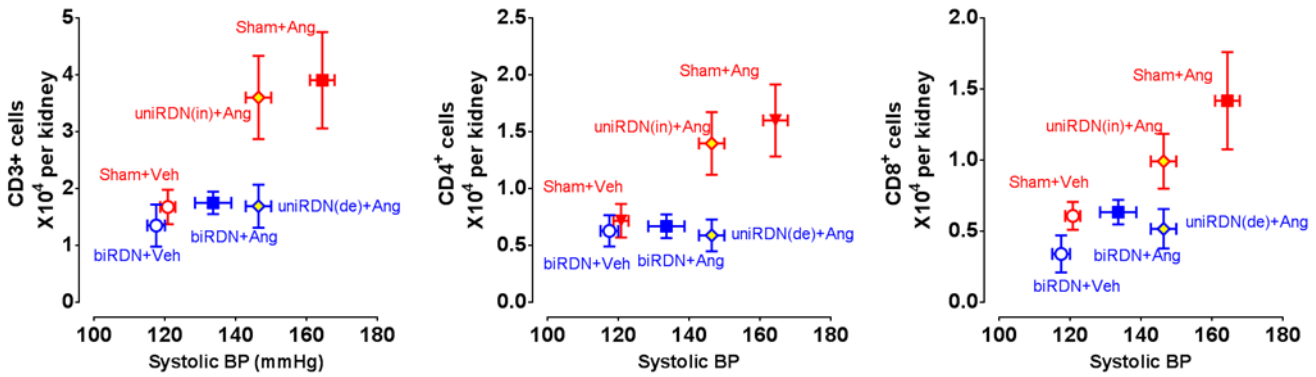

**Online Figure I: Effects of renal denervation on adjacent lymph nodes and adrenal glands.** The neuronal marker beta 3 tubulin was visualized in the lymph nodes adjacent to the kidneys of mice by confocal microscopy (A). Norepinephrine and epinephrine content in adrenal glands were measured by HPLC (B). Data were analyzed using unpaired t tests, n=5 in each group. No statistical significant difference was detected between groups.



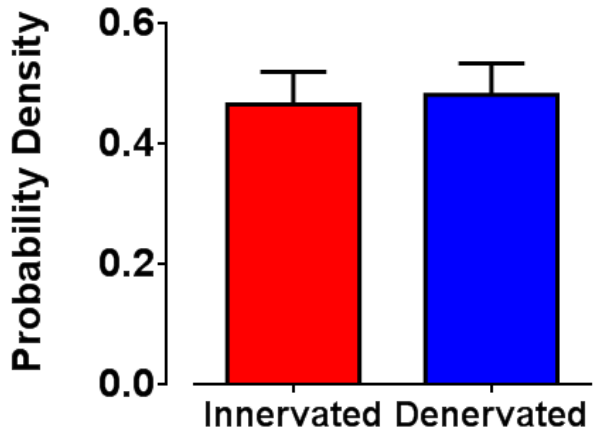

**Online Figure II:** Effect of renal denervation on aortic inflammation in response to angiotensin II. Mice underwent bilateral renal denervation or sham surgery and then received angiotensin II (490 ng/kg/min) for 14 days. Aortas were harvested for flow cytometry and total leukocytes (A), T cells (B) and $CD4^+$ and $CD8^+$ T cells (C and D) quantified using flow cytometry. Data were analyzed using one-way ANOVA, n=5 in each group. *P < 0.05, **P < 0.01, ***P<0.001.



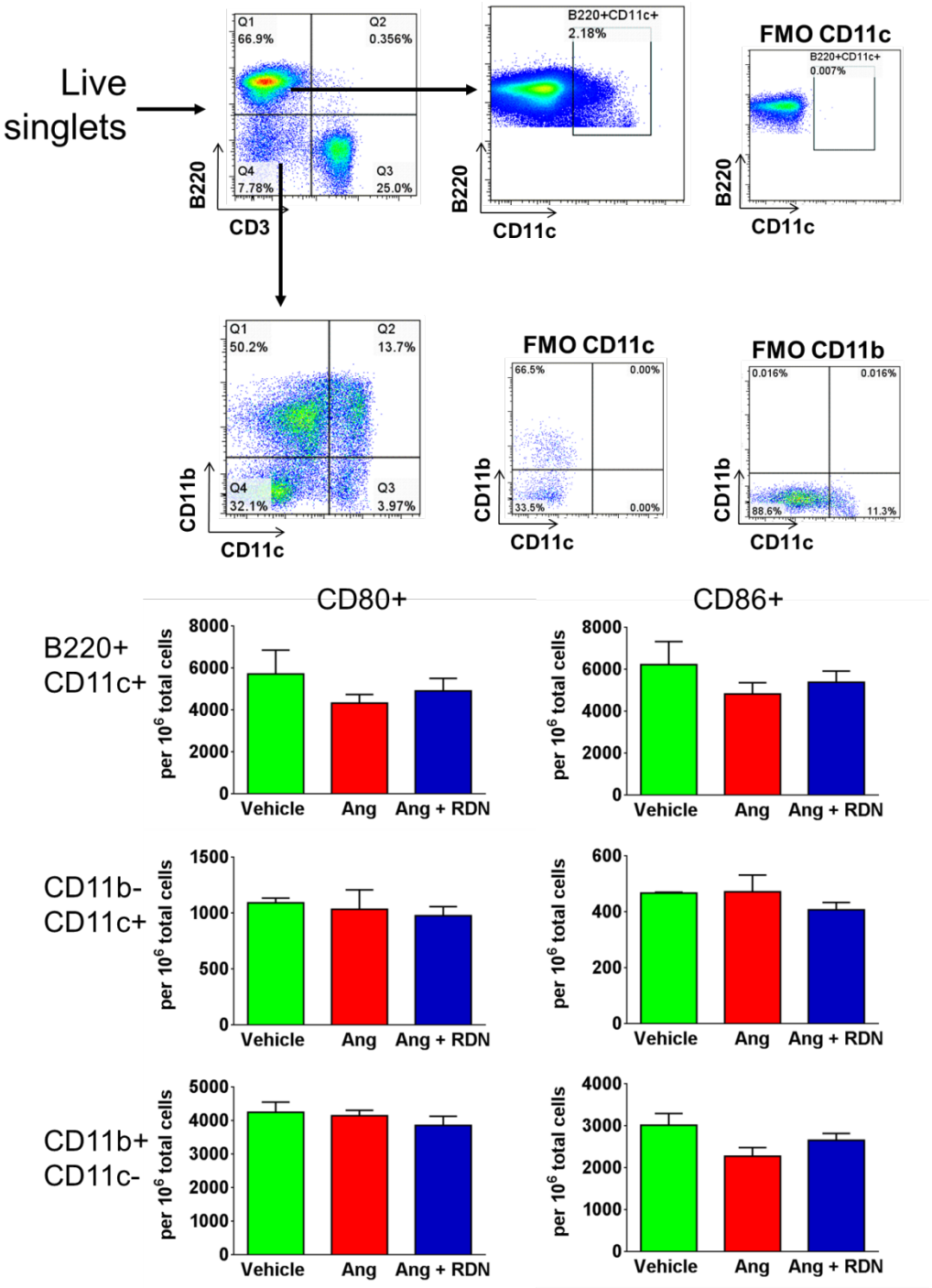

**Online Figure III: Gating strategy for detection of macrophages, dendritic cells and monocytes from spleen and kidney.**



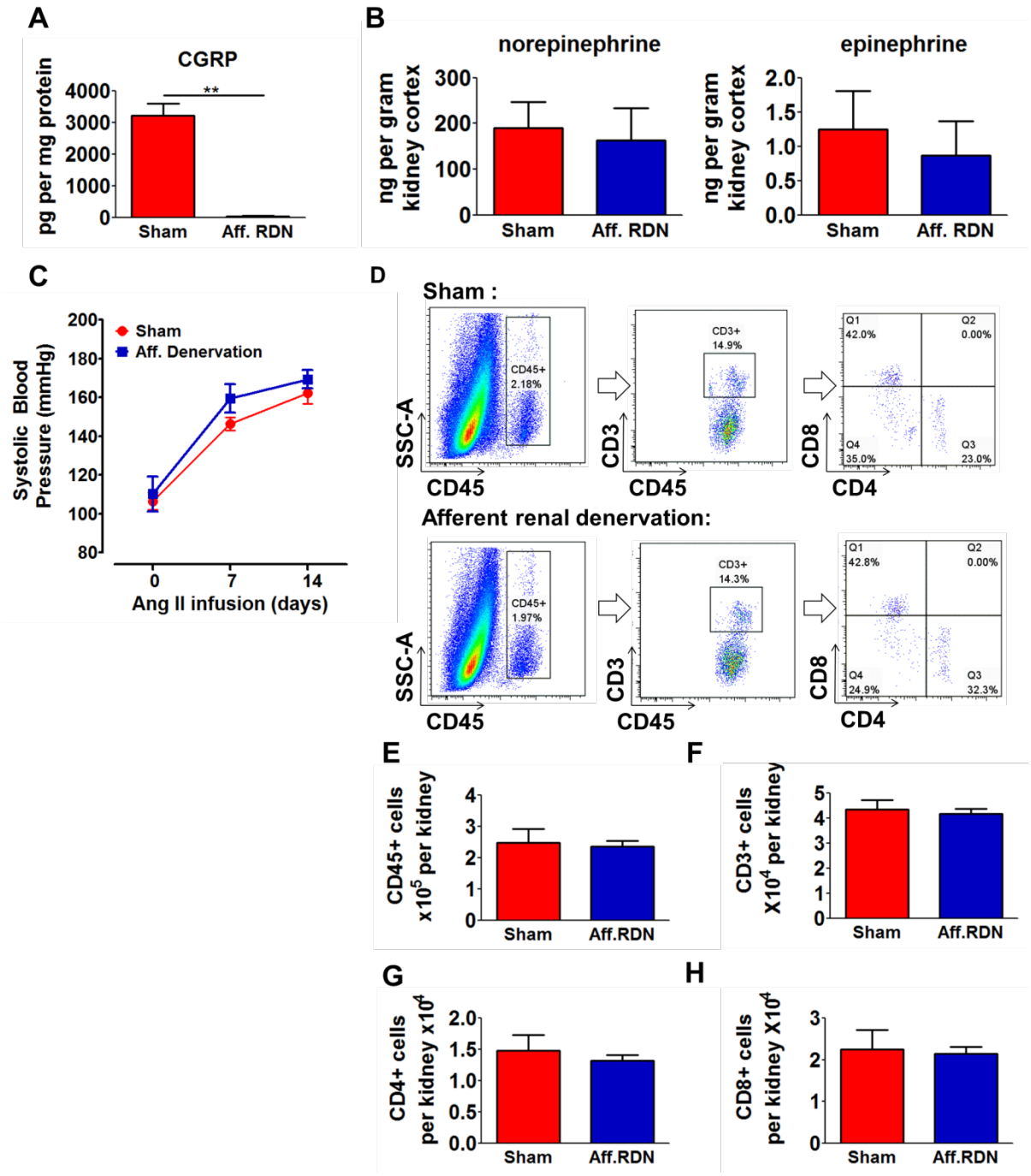

**Online Figure IV: Correlation between systolic pressure and renal T cell infiltration in mice after sham, unilateral (uniRDN) and bilateral renal denervation (biRDN).** Mice underwent either sham surgery, unilateral or bilateral renal denervation and subsequently received either vehicle (Veh) or angiotensin II (Ang) infusion for two weeks. In unilateral denervated mice, the innervated (in) and denervated (de) kidneys are plotted seperatedly. Flow cytometry was used to quantify total T cells (left panel), $CD4^+$ and $CD8^+$ T cells (middle and right panel).



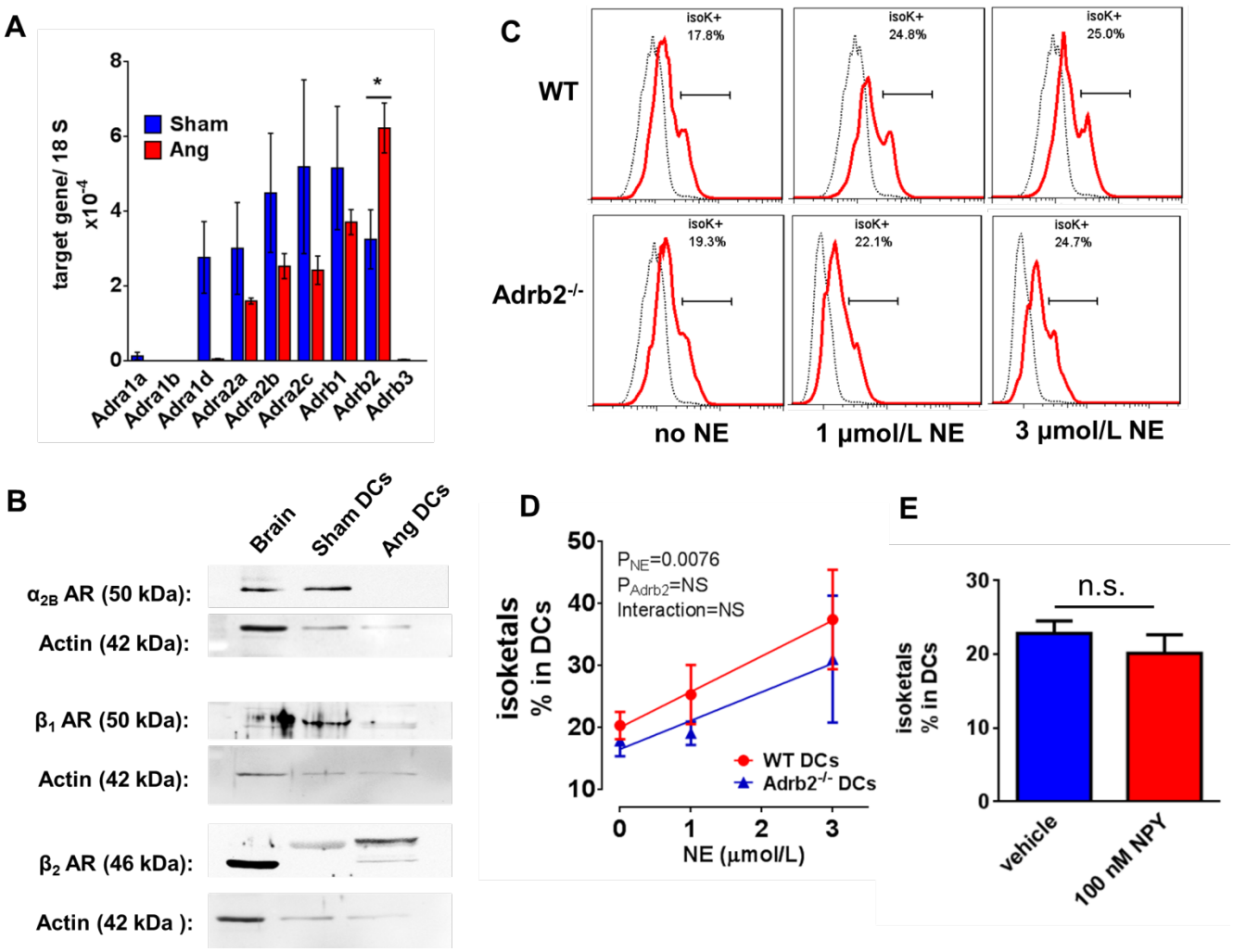

**Online Figure V: Effect of renal denervation on relative renal blood flow in mice with unilateral renal denervation after two-week angiotensin II infusion.** Renal MRI was performed as described in the supplemental method. Data were analyzed with paired t test, n=5. *P < 0.05, ***P < 0.001.



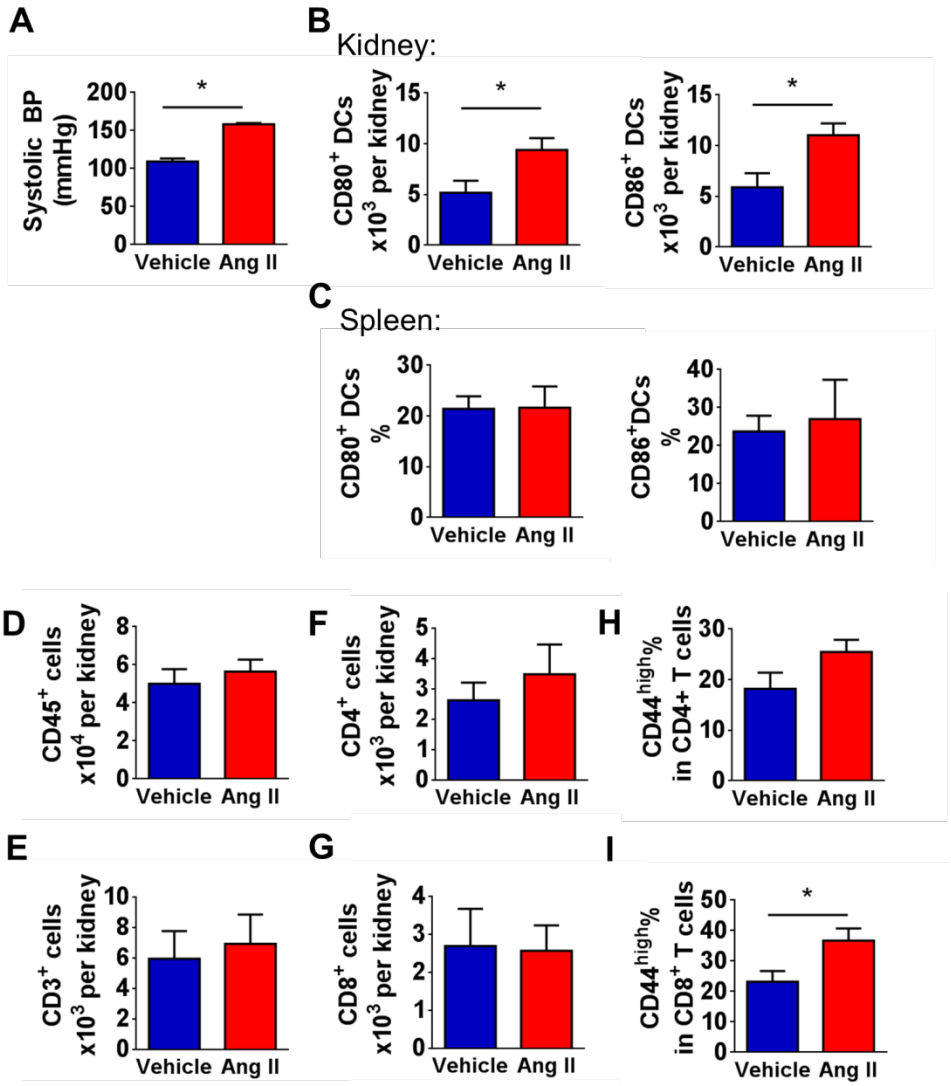

**Online Figure VI: Effects of renal denervation on activation of other antigen presenting cells in the spleen in mice with angiotensin II infusion**.




**Online Figure VII: Ablation of afferent renal nerves does not prevent hypertension or renal inflammation.** Selective ablation of afferent renal nerves by capsaicin treatment was validated by measurements of calcitonin gene related peptide (CGRP) (A). Renal norepinephrine and epinephrine contents were measured by HPLC as described in the methods (B). Systolic blood pressure was measured using the tail cuff method (C, n=8 for each group.) Flow cytometry of single cell homogenates was used to quantify renal infiltration of leukocytes and lymphocytes in the kidney (D-H). Data for panels A and B (n=5 per group) were analyzed using paired t test. Data in panel C was measured using two-way ANOVA. Data in panels E – H were analyzed by unpaired t tests.




**Online Figure VIII: Effects of sympathetic signaling on isoketal-protein adduct formation in dendritic cells.** DCs were magnetically isolated from spleens of mice that received vehicle or angiotensin II for two weeks. mRNA expression of adrenergic receptor (AR) subtypes were measured by realtime PCR (n=3). Protein levels of $\alpha_{2B}$, $\beta_1$ and $\beta_2$ adrenergic receptors in DCs were determined by Western blot and mouse brain was used as a positive control (B). DCs were derived from bone marrow of either wild type or $\beta$2AR$^{-/-}$ mice using GM-CSF and IL-4, and cultured with norepinephrine *in vitro* for 7 days (n=5 per group). In other experiments (E) wild type bone marrow-derived DCs were cultured in the presence of neuropeptide Y (n=4 per group). Isoketal-protein adducts were quantified by flow cytometry. Data in panels A and E were analyzed with t test. Data in panel D were analyzed using two-way ANOVA.




**Online Figure IX: Effect CCR7 deficiency on hypertension, dendritic cell maturation and renal inflammation.** CCR7$^{-/-}$ mice received angiotensin II (490 ng/kg.min) for two weeks. Blood pressure was measured by tail cuff (A). Flow cytometry was used to determine surface expression of CD80 and CD86 on DCs from the kidney (B) and spleen (C) and the renal presence of total leukocytes (D), T cells (E to G), and memory T cells (H and I). Data were analyzed using t tests, n=5 in each group. *P < 0.05.